# Phosphodiesterase 2 inhibition preferentially promotes NO-guanylyl cyclase-cGMP signaling to reverse the development of heart failure

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**Heart failure (HF) is a shared manifestation of several cardiovascular pathologies, including hypertension and myocardial infarction, and a limited repertoire of treatment modalities entails the associated morbidity and mortality remain high. Impaired nitric oxide (NO)-guanylyl cyclase (GC)-cyclic guanosine-3',5'-monophosphate (cGMP) signaling, underpinned in part by up-regulation of cyclic nucleotide-hydrolyzing phosphodiesterase (PDE) isozymes, contributes to the pathogenesis of HF, and interventions targeted to enhancing cGMP have proven effective in pre-clinical models and patients. Numerous PDE isozymes coordinate the regulation of cardiac cGMP in the context of HF; PDE2 expression and activity is up-regulated in experimental and human HF, but a well-defined role for this isoform in pathogenesis has yet to be established, certainly in terms of cGMP signaling. Herein, using a selective pharmacological inhibitor of PDE2, BAY 60-7550, and transgenic mice lacking either NO-sensitive guanylyl cyclase $\alpha_1$ (GC-1$\alpha^{-/-}$) or natriuretic peptide-responsive guanylyl cyclase-A (GC-A$^{-/-}$), we demonstrate the blockade of PDE2 promotes cGMP signaling to offset the pathogenesis of experimental HF (induced by pressure-overload or sympathetic hyper-activation), reversing the development of left ventricular hypertrophy, compromised contractility and cardiac fibrosis. Moreover, we show that this beneficial pharmacodynamic profile is maintained in GC-A$^{-/-}$ mice, but absent in animals null for GC-1α or treated with a NO synthase inhibitor, revealing that PDE2 inhibition preferentially enhances NO/GC/cGMP signaling in the setting of HF to exert a wide-ranging protection to preserve cardiac structure and function. These data substantiate the targeting of PDE2 in HF as a tangible approach to maximize myocardial cGMP-signaling and enhancing therapy.**

Nitric oxide | Natriuretic peptide | Cyclic GMP | Phosphodiesterase | Heart failure

## INTRODUCTION

Left ventricular hypertrophy (LVH) and subsequent heart failure (HF) are common to many cardiovascular disorders, including hypertension and myocardial infarction, and after age represent the most significant independent risk factors for cardiovascular morbidity and mortality(1). Current therapy focuses on reducing excess fluid load (e.g. diuretics) and blocking neuro-hormonal pathways (e.g. β-blockers, angiotensin converting enzyme [ACE] inhibitors)(2). Unfortunately, these interventions do not offer a cure but only slow deterioration in LV function. Consequently, HF is still associated with a 5 year survival rate of ~50%; the disorder therefore represents a clear unmet medical need.

Generation of the second messenger cyclic GMP (cGMP) by activation of NO- ($\alpha_1\beta_1$ [GC-1] and $\alpha_2\beta_1$ [GC-2])(3) and natriuretic peptide- (GC-A and GC-B)(4) sensitive guanylyl cyclases plays a key role in maintaining physiological cardiac contractility and integrity, and in offsetting the pathogenesis of LVH and HF(5). From a homeostatic perspective, endothelial (eNOS) and neuronal (nNOS) NO synthase appear to function in a complementary manner; eNOS-derived NO is thought to contribute to LV compliance, β-adrenergic inotropy, and amplifies parasympathetic innervation, whereas nNOS-generated NO regulates basal myocardial inotropy and lusitropy via inhibition of $I_{Ca}$, sympathovagal balance, and limits the activity of oxidases(6, 7). Indeed, in LVH such protective NO-mediated systems are depressed, in part due to diminished NO bioavailability and elevated GC-1/2 heme oxidation(8-10), driven by an increase in the production of reactive oxygen species, particularly by NADPH oxidase isoforms(11). Likewise, natriuretic peptides maintain cardiac structure and function in both physiological and pathological settings, as illustrated by the hypertrophic, fibrotic cardiac phenotype in transgenic animals lacking these mediators or cognate receptors, and the exacerbated response of such mice to cardiac stress (12-14).

From a pharmacological standpoint, NO donors, GC-1/2 ('sGC') stimulators and exogenously applied natriuretic peptides have been shown to decrease hypertrophy in cardiomyocytes and offset the development of HF in animal models and patients(15, 16). The common generation of cGMP and G-kinase activation affects a plethora of maladaptive, hypertrophic pathways including functional inhibition of $Ca^{2+}$ channels and $Ca^{2+}$ sequestration(17, 18), calcineurin/NFAT (nuclear factor of activated T-cells) signaling(19), blockade of regulators of G-protein signaling [RGS] proteins(20), transient receptor potential cation channel (TRPC)(21) and myosin binding protein (MBP)-C(22). This cardioprotective profile of cGMP in HF is exemplified

### Significance

**The morbidity and mortality associated with heart failure (HF) are unacceptably high. Cyclic guanosine-3',5'-monophosphate (cGMP) plays a key role in preserving cardiac structure and function, and therapeutically targeting cGMP in HF has shown promise in experimental models and patients. Phosphodiesterases (PDEs) metabolize and curtail the actions of cGMP (and cyclic adenosine-3',5'-monophosphate, cAMP) and increased PDE activity is thought to contribute to HF pathogenesis. Herein, we show that inhibition of one specific isoform, PDE2, enhances the salutary effects of cGMP in the context of HF, and that this beneficial action facilitates a distinct pathway, driven by nitric oxide, that is impaired in this disorder. These observations validate PDE2 inhibitors as a demonstrable means of boosting cardiac cGMP and advancing HF therapy.**

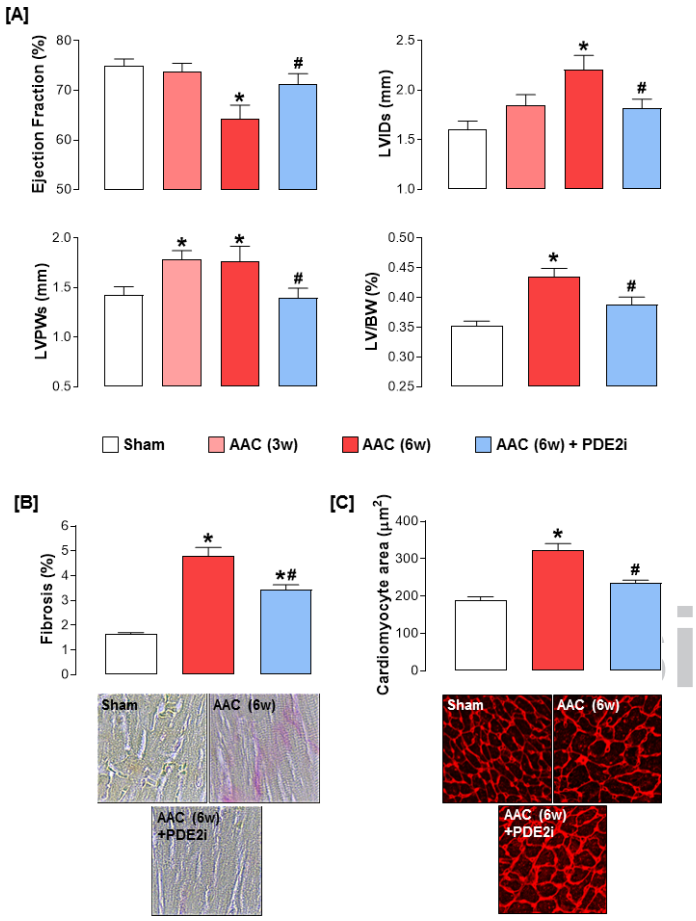


**Fig. 1. PDE2 inhibition reverses experimental HF in response to pressure-overload.** ***(A)*** Echocardiographic indices of heart structure & function, ***(B)*** cardiac fibrosis, and ***(C)*** cardiomyocyte size in sham mice and animals undergoing abdominal aortic constriction (AAC) for 3 weeks (3w) or 6 weeks (6w) in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 3w). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test. *p<0.05 versus sham, #p<0.05 v AAC (6w). n=12-18.

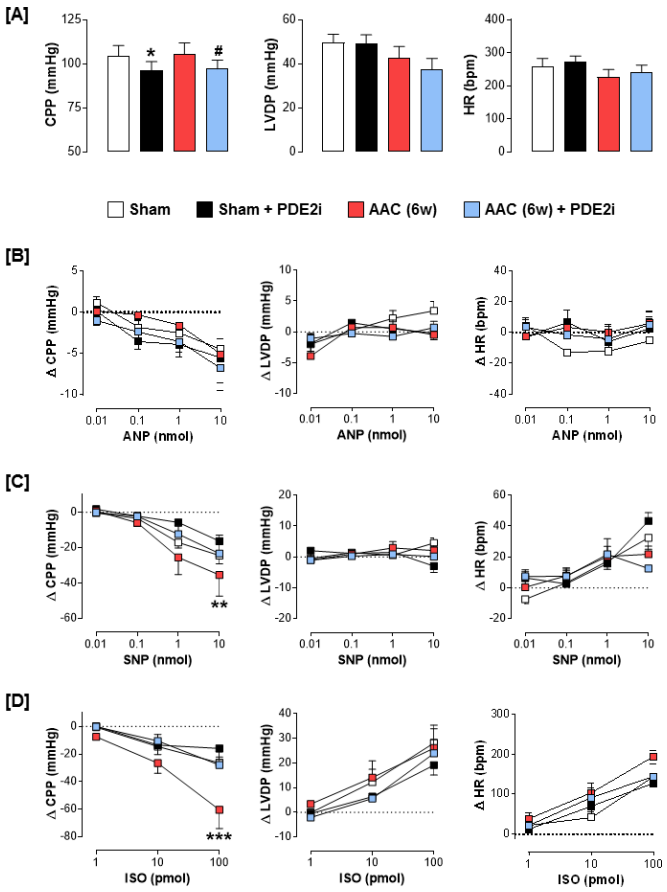


**Fig. 2. The beneficial effect of PDE2 inhibition in pressure overload-induced HF is not underpinned by acute changes in cardiac function.** ***(A)*** Coronary perfusion pressure (CPP), left ventricular developed pressure (LVDP) and heart rate (HR) in WT sham mice or animals subjected to 6 weeks of abdominal aortic constriction (ACC) in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 3w). CPP, LVDP and HR in sham mice and animals subjected to 6w AAC in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 3w) following the addition of increasing doses of ***(B)*** ANP, ***(C)*** SNP, and ***(D)*** isoproterenol (ISO). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test ***(A)*** or two-way ANOVA ***(B-D)***. *p<0.01 v sham or #p<0.05 v AAC (6w; ***A***) and **p<0.01, ***p<0.001 versus AAC (6w; ***C-D***). n=6-12.

by positive clinical outcomes with the dual neutral endopeptidase (an enzyme that inactivates natriuretic peptides) inhibitor-angiotensin receptor blocker LCZ696(23) and the sGC stimulator vericiguat(15).

An alternative therapeutic strategy to augment cGMP signaling for cardioprotection is inhibition of phosphodiesterases (PDEs). Cardiac cGMP concentrations are dynamically regulated by GC-driven synthesis in cooperation with degradation mediated by PDEs, a family of enzymes that hydrolyze cGMP and its sibling cyclic adenosine-3'-5'-monophosphate (cAMP)(24). In the heart, PDE isozymes 1, 2, 5 and 9 are believed to exert the most influential effects on cGMP signaling(25-28). Of these, PDE1 is the major isoform expressed in the healthy myocardium(29), whereas PDE5 is only minimally expressed under physiological conditions but significantly up-regulated in the failing myocardium(30, 31). In HF, inhibition of cGMP-metabolizing PDEs 1, 5 & 9 slows pathogenesis(27, 28); indeed, upregulation of expression and/or activity of PDEs is thought to underpin the diminution of cGMP signaling characteristic of HF(32). Several small clinical studies support a beneficial role for PDE5i in HF(33-36), but the RELAX trial, a randomized, double-blinded evaluation of sildenafil in HF patients with preserved ejection fraction (HFpEF) showed no significant benefit(37). This disappointing outcome intimates that if the therapeutic activity of cGMP is to be harnessed optimally, interventions targeting alternate, or more likely multiple, cGMP synthetic and/or degradative pathways will be necessary.

A relatively unexplored mechanism that might address this therapeutic deficiency is PDE2. This 'cGMP-stimulated' PDE metabolizes both cGMP and cAMP, and possesses a GAF-B domain(38) within its N-terminus(24) that acts as a negative feedback loop to expedite cyclic nucleotide hydrolysis in the presence of cGMP (akin to PDE5). Three PDE2 splice variants have been identified and are expressed in a wide variety of cells and tissues including the heart, platelets and endothelium(24); moreover, PDE2 expression is increased in both animals and patients with HF(39). PDE2 isozymes are kinetically indistinguishable, but the 2A2 and 2A3 variants have a N-terminal membrane localization motif that results in a predominantly particulate distribution. This cellular localization seems key to the functioning of PDE2 in the heart due to compartmentalization of cGMP signaling in cardiomyocytes(26, 40) and has been speculated to play a key role in modulating the development of LVH and HF. For example, up-regulation of PDE2 expression and activity may be protective by opposing the effects of sympathetic activation via cAMP breakdown(39, 41, 42). Indeed, PDE2 over-expression

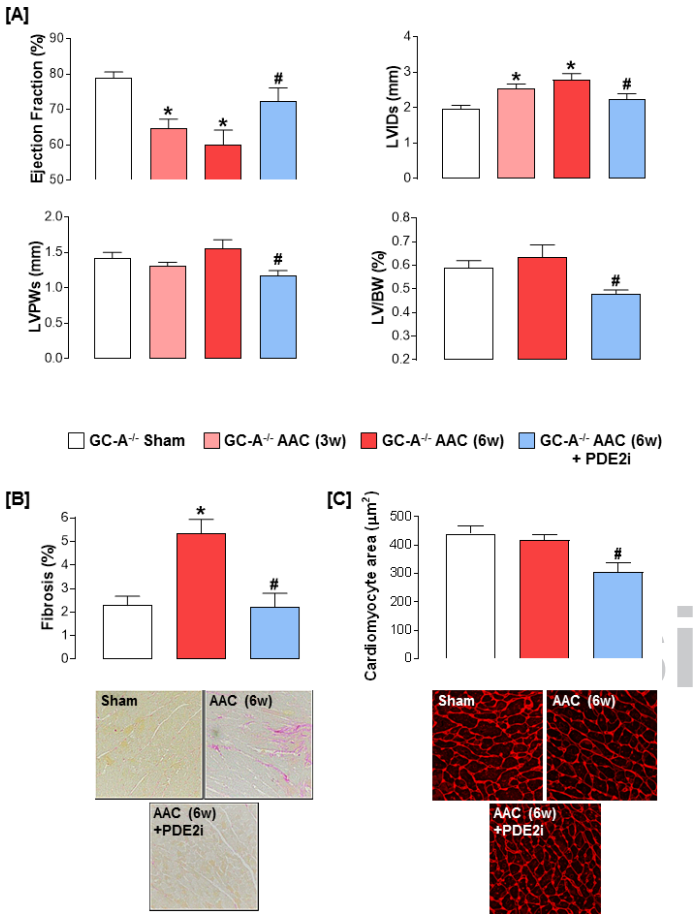


**Fig. 3. PDE2 inhibition reverses pressure overload-induced HF in GC-A$^{-/-}$ mice.** ***(A)*** Echocardiographic indices of heart structure & function, ***(B)*** cardiac fibrosis, and ***(C)*** cardiomyocyte size in sham mice and animals undergoing abdominal aortic constriction (AAC) for 3 weeks (3w) or 6 weeks (6w) in the absence and presence of BAY 60-7550 (10mg/kg/day; p.o.; initiated at 3w). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test. *p<0.05 v sham; #p<0.05 v AAC (6w). n=6-12.

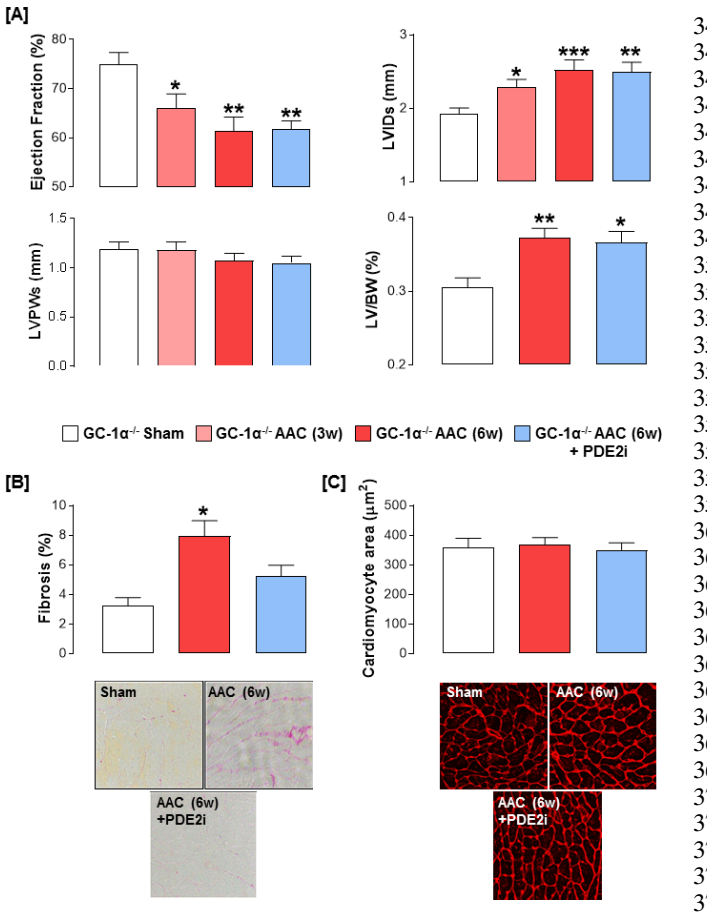


**Fig. 4. Obligatory role of GC-1/NO signaling in the beneficial effects of PDE2 inhibition in HF.** ***(A)*** Echocardiographic indices of heart structure & function, ***(B)*** cardiac fibrosis, and ***(C)*** cardiomyocyte size in sham mice and animals undergoing abdominal aortic constriction (AAC) for 3 weeks (3w) or 6 weeks (6w) in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 3w). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001 v sham. n=6-12.

has been reported to lower HR and maintain LV function and subdue arrhythmogenesis in experimental MI(43). Alternatively, PDE2 inhibition can prevent the hydrolysis of a localized pool of cAMP leading to PKA-dependent phosphorylation of NFAT and an anti-hypertrophic response(44). However, these studies have focused primarily on modulation of cAMP levels (i.e. PDE2 as a 'cAMP-hydrolase') of which chronic, widespread increases are known to cause higher mortality in HF patients(45). In contrast, there is a paucity of *in vivo* evidence evaluating the influence of PDE2-mediated cGMP hydrolysis in LVH and HF, although in the setting of pulmonary hypertension (PH) our recent work has highlighted the beneficial effects of PDE2 inhibition in diminishing the vascular remodeling and right ventricular hypertrophy (RVH)(46). Herein, we address this deficit by establishing a cGMP-dependent cardioprotective effect of PDE2 in pre-clinical models of LVH and HF, and identify the guanylyl cyclase source of cGMP that drives this process.

## METHODS

All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, United Kingdom and had approval from a local ethics committee. Animals were housed in a temperature-controlled environment in a 12-hour light–dark cycle. Food and water were accessible *ad libitum.*

**Genotyping**

Genomic DNA was prepared from ear biopsies for analysis by polymerase chain reaction (PCR) using standard cycling parameters using the forward and reverse primers stated in ***SI Appendix, Table S1***, as we have described previously(47).

**Generation of GC-1α knockout mice**

This novel GC-1α$^{-/-}$ strain was developed at Pfizer Inc. Briefly, heterozygous mouse embryonic stem cells containing one GC-1α$^{-/-}$ allele modified by homologous recombination were obtained from Deltagen (San Carlos, CA). Long distance PCR was used to generate a 5.7 kb 5' homology arm and a 3.6 kb 3' homology arm flanking a 5.3 kb deletion encompassing exons 6 and 7 of the mouse GC-1α gene (GenBank accession number NM_021896). Exons 6 and 7 were replaced with a Lac-Z/Neo$^{r}$ selection cassette, disrupting the 3' end of the cyclase domain (***SI Appendix, Figure S1***). Mouse 129P2/OlaHsd (E14) cells were transformed by established techniques(48) and G418-resistant colonies were selected. Expanded clones were screened via long distance PCR and Southern analysis. One clone with the correctly modified GC-1α allele was used to generate the mouse line via standard blastocyst injection(48).

Successful deletion of GC-1α was confirmed by immunoblot, assay of enzyme activity in lung homogenates, functional vascular pharmacology in response to NO-donor, and measurement of mean arterial blood pressure. (***SI Appendix, Figure S1***). The body weight of WT and GC-1α$^{-/-}$ mice did not differ significantly at the time of experimentation (WT: 30.92±0.63g, GC-1α$^{-/-}$: 30.95±0.65g; P>0.05; n=10).

Expression: Lungs were homogenized ice-cold 50 mM Tris pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol containing protease inhibitor cocktail (Roche) using an Ultra Turrax T8 disperser (IKA Works Inc.). Homogenates were centrifuged at 1000 x g for 30 min at 4°C, the supernatants were collected and protein concentration determined using the Bio-Rad method according to the manufacturer's instructions. 20 μg of lung supernatant protein was separated by 7% SDS-PAGE using Novex Tris-Acetate gel system (Invitrogen) and proteins were transferred to nitrocellulose membrane (Invitrogen). Staining with Ponceau S red (Sigma) was

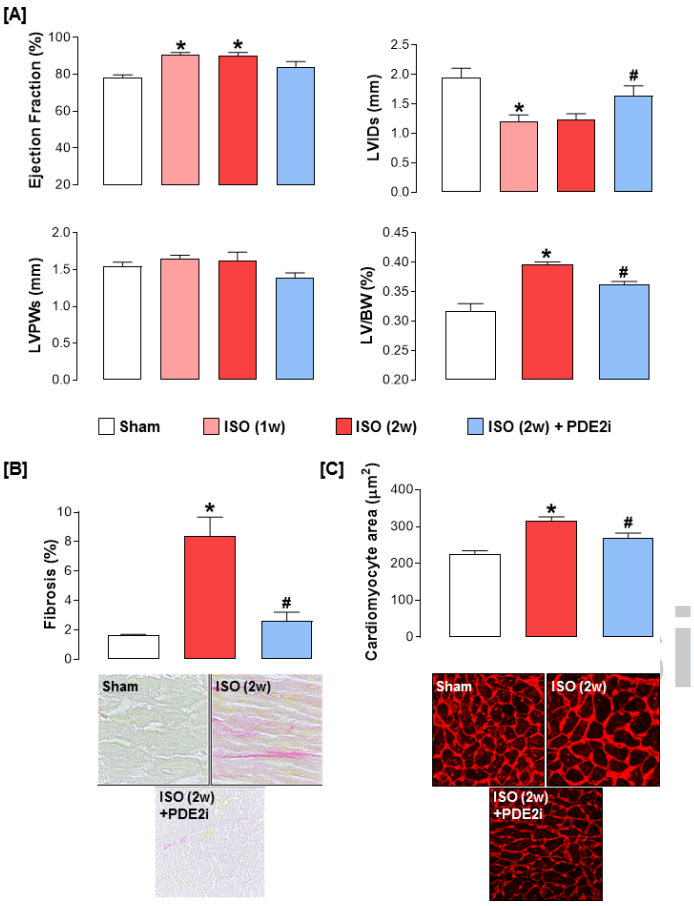


**Fig. 5. PDE2 inhibition reverses experimental HF in response to sympathetic hyperactivation.** ***(A)*** Echocardiographic indices of heart structure & function, ***(B)*** cardiac fibrosis, and ***(C)*** cardiomyocyte size in sham mice and animals administered isoproterenol (20mg/kg/day; s.c.) for 2 weeks in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 1w). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test. *p<0.05 versus sham, #p<0.05 v AAC (6w). n=6-12.

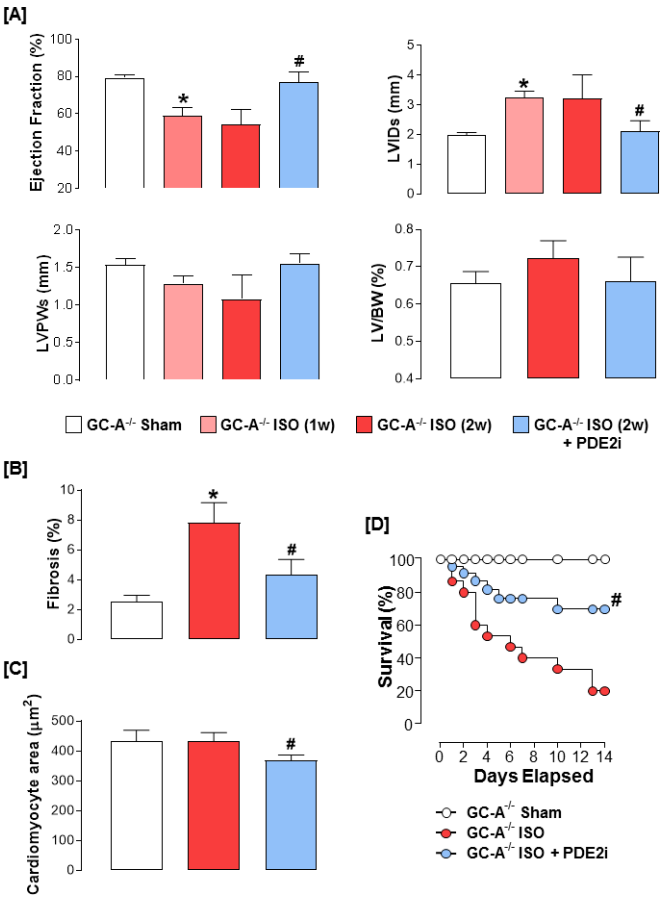


**Fig. 6. PDE2 inhibition reverses sympathetic hyperactivation-induced HF in GC-A$^{-/-}$ mice.** ***(A)*** Echocardiographic indices of heart structure & function, ***(B)*** cardiac fibrosis, and ***(C)*** cardiomyocyte size in sham mice and animals administered isoproterenol (20 mg/kg/day; s.c.) for 2 weeks in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 1w). ***(D)*** Survival in sham mice and animals administered isoproterenol (20 mg/kg/day; s.c.) for 2 weeks in the absence and presence of BAY 60-7550 (10mg/kg/day; p.o.; initiated at day 0). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test (***A-C***) or as a Kaplan-Meier survival plot (***D***). *p<0.05 v sham; #p<0.05 v AAC (6w; ***A-C***) or GC-A$^{-/-}$ + ISO (***D***). n=6-12.

used to confirm uniformity of protein loading. Membranes were blocked with 5% milk (Santa Cruz Biotechnology) dissolved in TBS-Tween 20, pH 7.4 (ScyTek Laboratories) for 1.5 h at room temperature. For the detection of GC-1α, membranes were incubated with polyclonal rabbit anti-GC-1α serum (Alexis Biochemicals, 1:2000 dilution) and for the GC-1β subunit detection with polyclonal rabbit anti-GC-1β affinity isolated antibody (1:1000 dilution, Alexis Biochemicals) for 1 h at room temperature. Membranes were washed with TBS-Tween 20, pH 7.4 buffer and incubated with 2° antibody (goat anti-rabbit HRP-conjugated IgG, 1:2500 dilution; Pierce) for 1 h at room temperature and followed by four washes in TBS-Tween, pH 7.4. Dilutions of primary and secondary antibodies were prepared in TBS-Tween 20 buffer. Antibody reactivity was detected using SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, Waltham, MA, USA) and Kodak film (Sigma, St. Louis, MO, USA).

Activity: Assays were performed in 100 μL containing of 50 mM Tris, pH 7.4; 1 mM DTT; 0.2 mM GTP; 5 mM $MgCl_2$, 0.5 mM IBMX, and 1-10 μg of lung extract protein. DETA-NONOate (100 μM) was included as specified. Reactions were started by addition of substrate, incubated for 60 min at 37°C and terminated with the addition of 20 mM EDTA. Cyclic GMP was then quantified using the CatchPoint™ cGMP Fluorescent Assay Kit (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Vascular reactivity: The vascular reactivity of mouse thoracic aortic vascular ring preparations was determined using classical tissue bath pharmacology, as we have described previously(49).

Blood pressure & heart rate: Blood pressure was recorded in conscious freely moving mice using radiotelemetric transmitters (TA11PA-C10, Data Sciences International, Minneapolis, USA) implanted into the aortic arch, as we have described previously(49).

**Pressure overload-induced HF**

Pressure-overload LVH and cardiac dysfunction were induced by performing abdominal aortic constriction (AAC) at the suprarenal level. Male wild type (WT), GC-1α$^{-/-}$ (as described above) and GC-A$^{-/-}$ (kind gift of Prof. O. Smithies, University of North Carolina) mice (21–23 g; offspring of heterozygote parents to enable use of corresponding WT littermate controls) were anesthetized (1.5% isoflurane in $O_2$), body temperature maintained at 37°C and the analgesic buprenorphine (0.1 mg/kg) administered (i.m.). An incision was made in the abdominal cavity, and the abdominal aorta was separated from the surrounding tissue at the suprarenal level. Aortic constriction was performed by tying a 4-0 surgical thread against a 25-gauge needle between the superior mesenteric and renal arteries. This produces a 30% constriction of the luminal diameter(50). For sham operations, the 4-0 surgical thread was passed under the aorta and removed without tying it against the needle. In some studies, WT mice undergoing AAC were administered the NOS inhibitor L-N$^G$-nitroarginine methylester (L-NAME; 100 mg/kg/day) via the drinking water for 7 days prior to and during the AAC model.

**Chronic sympathetic activation-induced HF**

Sustained sympathetic activation is characteristic of HF and can be recapitulated in pre-clinical models using chronic dosing with the β-agonist isoproterenol(51). Male WT, GC-1$^{-/-}$ and GC-A$^{-/-}$ mice (24-25 g;) were infused subcutaneously with isoproterenol (20 mg/kg/day, 14 days; Sigma-Aldrich, Poole, UK) via osmotic mini-pumps (model 1002, Alzet, Cupertino, CA). Saline containing 0.5% ascorbic acid was used as the solvent for isoproterenol to avoid catecholamine oxidation over time.

**Treatment regimens**

Animals were assigned randomly to receive the selective PDE2 inhibitor BAY 60-7550(52) (10 mg/kg/day; kind gift of Dr. J.-P. Stasch & P. Sandner, Bayer AG, Wuppertal, Germany) or vehicle control (0.5% carboxycellulose +10% polyethylene glycol) by daily oral gavage, initiated 3 weeks after AAC

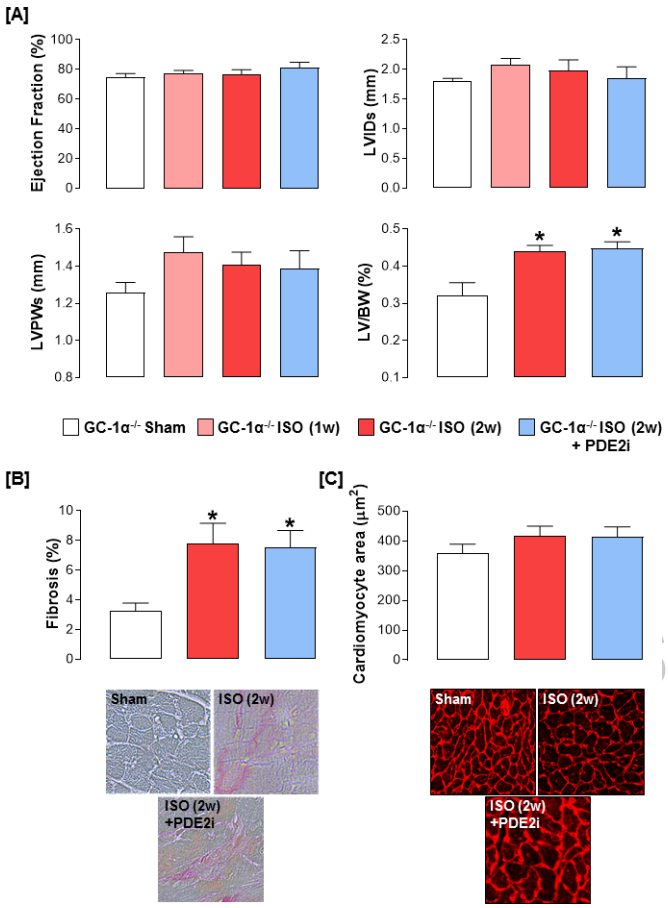


**Fig. 7. Obligatory role of GC-1/NO signaling in the beneficial effects of PDE2 inhibition in sympathetic hyperactivation-induced HF.** ***(A)*** Echocardiographic indices of heart structure & function, ***(B)*** cardiac fibrosis, and ***(C)*** cardiomyocyte size in sham mice and animals administered isoproterenol (20 mg/kg/day; s.c.) for 2 weeks in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 1w). Data are expressed as mean±sem and analyzed by one-way ANOVA with Bonferroni post hoc test. *p<0.05 v sham. n=6-12.

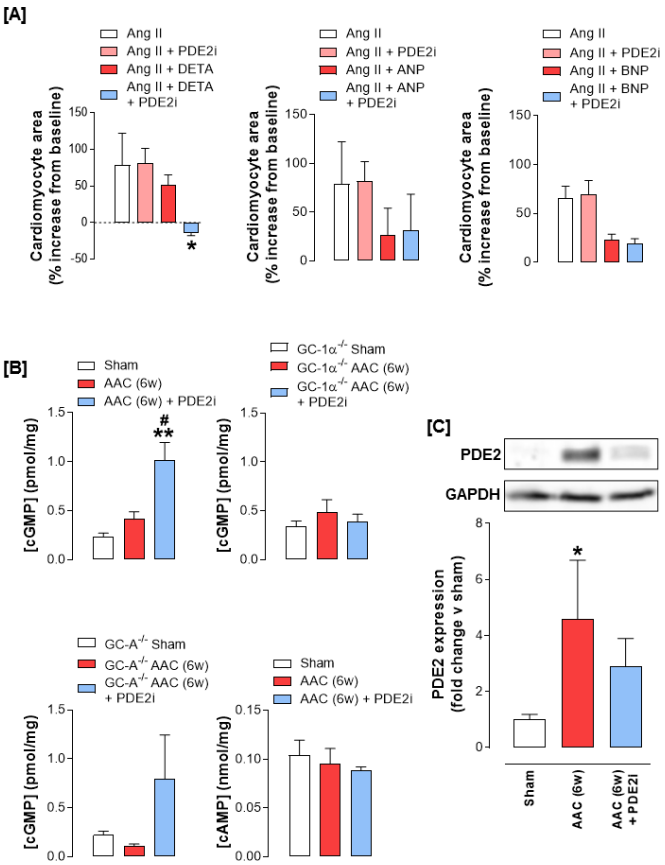


**Fig. 8. PDE2 inhibition augments cardiac cGMP, but not cAMP, levels in vivo and promotes the anti-hypertrophic effect of NO, but not ANP, in isolated cardiomyocytes.** ***(A)*** Cardiomyocyte area in cells isolated from neonatal WT mice treated with AngII (100 nM) for 24 h in the absence and presence of the NO donor DETA-NONOate (DETA; 10 μM), atrial natriuretic peptide (ANP; 1 μM) or brain natriuretic peptide (BNP; 1 μM) under basal conditions or following treatment with BAY 60-7550 (100 nM). ***(B)*** Cyclic nucleotide levels in whole heart homogenates from sham mice or animals subjected to abdominal aortic constriction (AAC) for 6 weeks in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.). ***(C)*** Immunoblot analysis of PDE2A expression in whole heart homogenates from mice subjected to abdominal aortic constriction (AAC) for 6 weeks in the absence and presence of BAY 60-7550 (10 mg/kg/day; p.o.; initiated at 3w). Data are expressed as mean±sem with analysis by one-way ANOVA with Bonferroni post hoc test. *p<0.05 v AngII + DETA-NONOate (***A***), **p<0.01 v sham and #p<0.0 v AAC (***B***), *p<0.05 v sham (***C***). n=6.

or 1 week after isoproterenol infusion (i.e. after the HF phenotype had developed).

***In vivo* cardiac functional assessments**

*In vivo* cardiac morphology and function were assessed by M-mode echocardiography using a VisualSonics Vevo 770 echocardiographic system and a 30 MHz transducer. Mice were anesthetized (1.5% isoflurane in $O_2$) and body temperature maintained at 37°C. LV internal diameter (LVID), and LV posterior wall thicknesses (LVPW) at diastole (d) and systole (s) were measured from short-axis M-mode images. LV ejection fraction (EF%) was calculated as follows: LVEF% = $[(LVIDd)^3 - (LVIDs)^3]/(LVIDd)^3 \times 100$; LV fractional shortening (FS%) was calculated as follows: LVFS% = (LVIDd – LVIDs)/LVIDd × 100. Values were averaged from 3 beats.

**Langendorff isolated heart preparations**

*Ex vivo* cardiac function and the effects of acute PDE2 inhibition were evaluated in murine hearts set-up in Langendorff mode, as we have described previously(53). Acute changes in these parameters were recorded in response to bolus injections of ANP (0.01-10 nmol), the NO-donor sodium nitroprusside (SNP; 0.01-10 nmol), the endothelium-dependent vasodilator acetylcholine (ACh; 0.1-1.0nmol), and the β-agonist isoproterenol (ISO; 1-100 pmol) in the absence and presence of BAY 60-7550 (100 nM).

**Histology staining and imaging**

The isolated left ventricles were cut transversely below the mitral valves, fixed in 10% formalin for 24h, then stored in 70% alcohol before embedding in paraffin wax and sectioning.

**Wheat germ agglutinin fluorescence staining**

Ventricular myocyte size was determined by staining heart sections with a fluorescent cell membrane antibody, wheat germ agglutinin alexafluor 647(Molecular Probes, Invitrogen, UK) and mounted with Prolong gold DAPI mountant as per standard immunohistochemistry protocols. Images were taken on a Zeiss 710 confocal microscope and the cardiomyocyte size analyzed with Image J (NIH). Cardiomyocyte size was estimated with investigator blinded to treatment type and as an average of approximately 400 cells per heart.

**Picrosirius red staining**

Tissue slides were dewaxed, rehydrated and stained using a Picrosirius Red Stain kit following the manufacturer's instructions (Polysciences, Inc. Warrington, PA, USA. A Nikon Eclipse TS100 microscope (Nikon UK Limited, Surrey, UK) was used to capture images of the stained slides. Images were analysed by threshold analysis using Image J with investigator blinded to treatment type.

**Primary cardiomyocyte isolation and culturing**

Primary cardiomyocytes were isolated using the Pierce primary cardiomyocyte isolation kit (Thermofisher Scientific, Crawley, UK) with minor modifications. Briefly, individual neonatal hearts from 1-3 day old mice were minced, treated with Cardiomyocyte Isolation Enzyme 1 (with papain) and Cardiomyocyte Isolation Enzyme 2 (with thermolysin) The resulting isolated CMs were seeded into gelatin (0.1%) pre-coated 12-well plates, and incubated in DMEM for three days till confluent. The cells were then serum starved for 24 h before treatment with Angiotensin (Ang) II (1μM) for 24 hours in the absence and presence of the NO donor DETA-NONOate (DETA; 10 μM), atrial natriuretic peptide (ANP; 1 μM) or brain natriuretic peptide (BNP; 1 μM) under basal conditions or following treatment with BAY 60-7550 (100 nM). Light microscopy images of beating cardiomyocytes were taken at

0 h (baseline) and 24 h. Image J was used to analyze the cardiomyocyte size. An average of 30 cells per heart, and 5-7 hearts per treatment were analyzed.

**Quantitative RT-PCR, immunoblotting & immunohistochemistry**

Whole hearts were homogenized using QIAshredder technology and RNA was extracted using a standard extraction kit (Qiagen, UK). RNA was quantified using a NanoDrop spectrophotometer (Thermofisher Scientific, MA, USA) and 1 µg converted to cDNA by reverse transcription (High Capacity RNA-to-cDNA Kit; Applied Biosystems, Life technologies Ltd, UK). Specific primers for PDE2A(54), hypertrophic or fibrotic markers and housekeeping genes RLP-19 and β-actin (300 nM; detailed in ***SI Appendix, Table S2***) were added to cDNA template and SyBr Green quantitative PCR mix (Quantitect Sybr green kit, Qiagen, UK). 20 ng of cDNA from each sample was amplified using quantitative real-time PCR over 40 cycles (initial denaturation: 10 min at 95°C; cycling: 45 cycles, 10 s at 95°C, 15 s at 57°C, and 5 s at 72°C; melt: 68-90°C). mRNA expression was analyzed by expressing the cycle threshold (Ct) value as $2^{-\Delta\Delta Ct}$ and normalized to both housekeeping genes (RPL-19 and β-actin).

PDE2A protein expression was determined by immunoblot using primary anti-PDE2A antibody (Santa Cruz Biotechnology, USA; 1:500) and secondary horse-radish peroxidase conjugated anti-goat IgG antibody (Santa Cruz Biotechnology; 1:10,000). Bands were quantitated by densitometry using ImageJ and normalized to the loading control, anti-GAPDH (1:50,000, Thermo Fisher Scientific, UK) and secondary antibody horse-radish peroxidase conjugated anti-mouse IgG (1:5,000; Dako, Cambridge, UK).

PDE2A localisation was assessed in heart sections by employing conjugated wheat germ agglutinin (WGA, Alexafluor 647, Molecular Probes, Invitrogen, UK) staining to outline cardiomyocyte boundaries. Slides were then double-stained with anti-PDE2A antibody (Santa Cruz Biotechnology, as above) and mounted with Prolong Gold DAPI mountant as per standard immunohistochemistry protocols. All slides were imaged using a Hamamatsu nanozoomer S60 (x40 magnification).

TUNEL (terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling) staining was conducted to assess levels of apoptosis in heart sections using a commercially-available kit (MK500; Takara Bio. Inc., Kusatsu, Japan). All slides were imaged using a Hamamatsu nanozoomer S60 (x20 magnification). TUNEL-positive cells and DAPI-stained nuclei were manually counted by quantitative image analysis using Image J. A positive control consisting of paraffin embedded sections of rat mammary gland were supplied as part of the kit.

**PDE2 activity**

PDE2 activity in whole heart homogenates was determined by assaying the concentration of cGMP and cAMP in the absence and presence of BAY 60-7550 (10 mg/kg/day) *in vivo* using commercially available kits (Direct cGMP and Direct cAMP; Enzo Life Sciences, Exeter, UK).

**Data analysis**

Results are expressed as mean±s.e.mean, and P<0.05 denotes significance. The n value denotes the number of animals used in each group and data were analyzed (GraphPad Prism version 5; GraphPad, La Jolla, CA, USA) using one-way or two-way ANOVA with a Bonferroni post hoc test as appropriate.

## RESULTS

### PDE2 inhibition reverses experimental HF in response to pressure-overload

Abdominal aortic constriction (AAC) brought about a sustained increase in MABP (***SI Appendix, Table S3***) and an archetypal HF phenotype as expected, comprising reduced contractility, LVH and LV dilatation (***Figure 1***). Cardiomyocyte size and Picrosirius red staining were also increased, indicative of a hypertrophied and fibrotic LV (***Figure 1 & SI Appendix, Figure S2***). Administration of BAY 60-7550 at week 3 resulted in a significant reversal of each of these indices of disease severity (***Figure 1 & SI Appendix, Figure S1***); indeed, in some cases the salutary effect of PDE2 inhibition was so pronounced that structure (e.g. cardiomyocyte size) and function (e.g. ejection fraction) were ostensibly returned to normal levels (***Figure 1***). Importantly, administration of BAY 60-7550 did not cause a significant reduction in MABP (***SI Appendix, Table S3***), excluding the possibility that PDE2 inhibition may lead to peripheral dilatation and a reduction in the cardiac overload, thereby indirectly dampening the HF phenotype; nor did BAY 60-7550 overtly alter HR (***Table 4***). These findings verify a substantial, multi-faceted protective effect of PDE2 inhibition in HF.

### The beneficial effects of PDE2 inhibition in pressure overload-induced HF are not underpinned by acute changes in cardiac function

One potential mechanism by which PDE2 inhibition might bring about a beneficial effect in the setting of HF, in terms of myocardial function, would be to exert an acute, positive inotropic effect thereby augmenting contractility. To rule this out, hearts from sham animals and mice undergoing AAC were isolated and set up in Langendorff mode to assess the effects of BAY 60-7550 *per se*, and on the actions of cGMP (i.e. NO and natriuretic peptides) and cAMP (isoproterenol) –elevating agents. Cardiac structure and function were recorded longitudinally by ECHO to ensure a HF phenotype was established in the mice undergoing AAC and this was corroborated by initial data from the hearts *ex vivo* which exhibited reduced contractility and impaired coronary function (***SI Appendix, Figure S3***). BAY 60-7550 caused a modest, yet significant fall in CPP in isolated hearts indicative of a subtle vasodilator effect (***Figure 2***). This vasorelaxant activity was present regardless of whether the hearts were isolated from sham animals or mice with HF. Addition of ANP caused a dose-dependent reduction in CPP but had little or no effect on contractility (i.e. LVDP) or HR (***Figure 2***). These responses were neither affected by the presence of BAY 60-7550 nor in failing hearts. Bolus delivery of SNP also caused a dose-dependent fall in CPP, was devoid of activity against LVDP, but tended to increase HR (***Figure 2***). Again, this profile was maintained in sham and failing hearts, and in the absence and presence of BAY 60-7550; the only exception being a reduction in vasodilation in response to PDE2 inhibition in hearts from AAC animals (***Figure 2***). Finally, isoproterenol caused a dose-dependent drop in CPP with a concomitant, overt increase in LVDP and HR, as anticipated of a cAMP-elevating agent. This coronary vasodilator activity and positive inotropic and chronotropic action were equivalent in sham and failing hearts and in the presence of BAY 60-7550 (apart from a slight reduction in coronary vasodilator activity with PDE2 inhibition in failing hearts; ***Figure 2***). BAY 60-7550 was unable to augment the positive inotropic and chronotropic actions of isoproterenol in this setting, establishing that metabolism of the pool of cAMP that regulates contractility and rate in sham or failing hearts is not a key role for PDE2; this dovetails well with a lack of effect of BAY 60-7550 on HR *in vivo* (***SI Appendix, Table S4***). These data clearly demonstrate that the favorable effects of BAY 60-7550 are not underpinned by an acute action on cardiac contractility but rather exerted via a more extensive, chronic influence on heart morphology (and potentially additional mechanisms including autonomic regulation(55, 56)).

### PDE2 inhibition reverses pressure overload-induced HF in GC-A$^{-/-}$ mice

We next sought to determine which arm of the guanylyl cyclase enzyme family was responsible for generating the pool of cGMP regulated by PDE2. Since our previous work in PH had highlighted a link between PDE2 and natriuretic peptide/GC-A-dependent cGMP synthesis, and the fact that genetic deletion of ANP, BNP or GC-A results in cardiac structural and functional deficits at baseline (12-14), we investigated the efficacy of BAY 60-7550 in pressure overload-induced HF in GC-A$^{-/-}$ mice. Unexpectedly, the beneficial effects of PDE2 inhibition on cardiac structure and function in AAC-driven HF in WT mice were maintained in GC-A$^{-/-}$ animals (***Figure 3***). As published previously, the GC-A$^{-/-}$ animals had marked intrinsic cardiac hypertrophy and dilatation (albeit with preserved ejection fraction; (***Figure 3***). Nonetheless, the reduction in contractility, increase in ventricular wall thickness & mass, and fibrotic burden were all significantly reversed in the presence of BAY 60-7550 (***Figure 3***); PDE2i also caused a reduction in cardiomyocyte size. Indeed, the positive pharmacodynamic profile of BAY 60-7550 was of a magnitude similar to that observed in WT mice (***Figure 1***). Such findings argue against a significant contribution of ANP/BNP signaling to the salutary actions of PDE2i in HF.

**Obligatory role of NO/GC-1α signaling in the beneficial effects of PDE2 inhibition**

As a result of the preserved pharmacology of BAY 60-7550 in GC-A$^{-/-}$ mice, we turned to exploration of a potential role for the NO/GC-1/cGMP. We took two approaches; first, using a transgenic strain with global deletion of GC-1α subunit (GC-1α$^{-/-}$) and second, treating WT animals with the NO synthase (NOS) inhibitor N$^{G}$-nitro-L-arginine methylester (L-NAME). The cardiovascular phenotype of the new GC-1α$^{-/-}$ strain generated herein mirrored that reported previously(57, 58) with loss of GC-1α protein, reduced vasorelaxant and cGMP-generating capacity of NO donors, and sex-dependent hypertension (***SI Appendix, Figure S1***).

GC-1α$^{-/-}$ mice exhibited a subtle cardiac phenotype at baseline, with a modest rise in LVID and increased cardiomyocyte size (albeit without a significant increase in (LV:BW ratio) in comparison to WT (***Figure 4***). In response to AAC, GC-1α$^{-/-}$ animals exhibited an almost identical phenotype to WT mice with reduced cardiac contractility, enlarged and dilated LV, and increase in fibrosis. Of note, the baseline cardiomyocyte size in GC-1α$^{-/-}$ animals was increased compared to WT and consequently AAC only marginally increased this index of hypertrophy (***Figure 4***). The mechanism underpinning the innate cardiomyocyte enlargement, but normal LV mass, in GC-1α$^{-/-}$ animals is not a result of cardiomyocyte loss since levels of apoptosis were not different to WT (***SI Appendix, Figure S4***); the reason for this intrinsic difference remains to be explained. Regardless, in sharp contrast to WT and GC-A$^{-/-}$ mice, PDE2i did little or nothing to improve the cardiac function in GC-1α$^{-/-}$ animals (***Figure 4***). To substantiate this critical involvement of NO/GC-1/cGMP signaling in the salutary effects of PDE2i, essentially identical studies were conducted in WT mice receiving L-NAME (***SI Appendix, Figure S5***). First, the consequences of pan-NOS inhibition *per se* on cardiac function following AAC were minimal, reflecting the phenotype of GC-1α$^{-/-}$ animals. Second, mirroring observations in GC-1α$^{-/-}$ mice, the pharmacodynamic benefit of BAY 60-7550 was completely absent, including effects to reverse the fibrotic burden that had been maintained in GC-1α$^{-/-}$ animals (***SI Appendix, Figure S5***). Notably, GC-1α expression tended to be up-regulated in response to pressure overload (***SI Appendix, Figure S2***). These data provide genetic and pharmacological verification that it is the NO-driven arm of cGMP signaling that is responsible for the positive effects of PDE2i in HF.

**PDE2 inhibition reverses experimental HF in response to sympathetic hyperactivation in a NO/GC-1α-dependent, but GC-A-independent, manner**

In order to demonstrate that PDE2 inhibition has a generic salutary effect in experimental models of HF the efficacy of BAY 60-7550 was also explored in isoproterenol-driven (i.e. sympathetic hyperactivation) cardiac dysfunction. Akin to pressure-overload, PDE2i brought about a multi-modal protection to reverse the cardiac contractile changes (in this instance more analogous to HF with preserved EF; HFpEF), LV hypertrophy and dilatation, and cardiac fibrosis (***Figure 5***). Furthermore, substantiating the concept that PDE2i augments NO/GC-1α signaling in HF, the positive outcome realized by BAY 60-7550 was maintained in GC-A$^{-/-}$ mice (***Figure 6***) but diminished or lost in GC-1α$^{-/-}$ animals (***Figure 7***). Interestingly, genetic deletion of GC-A transformed the predominantly HFpEF phenotype produced by isoproterenol to HF with severely impaired EF resulting in death in many animals (***Figure 6***), confirming the critical role(s) of endogenous natriuretic peptide signaling in preserving cardiac function in HF(14). Yet, BAY 60-7550 was still able to reverse this pathology and significantly improve mortality (***Figure 6***), proffering further evidence that targeting NO/GC-1 signaling is pharmacologically beneficial in HF; a thesis strengthened by the lack of efficacy of BAY 60-7550 in GC-1α$^{-/-}$ mice exposed to isoproterenol (***Figure 7***). A global comparison of the cardiac structural and functional indices in pressure overload-induced HF in WT, GC-A$^{-/-}$ and GC-1α$^{-/-}$ mice is depicted in ***SI Appendix, Figure S6***.

This model, characterized by excessive β-adrenoceptor/cAMP signaling also served as a means to tease out any cAMP-driven effects of PDE2 inhibition. In this regard, however, BAY 60-7550 did not exert any positive chronotropic effects (***SI Appendix, Table S4***).

**PDE2 inhibition boosts the anti-hypertrophic effect of NO, but not natriuretic peptides, in isolated cardiomyocytes**

To corroborate the importance of NO/GC-1/cGMP signaling to the HF phenotype, we followed up *in vivo* investigations with *in vitro* studies in isolated cardiomyocytes. Here, addition of NO, ANP and BNP reduced the hypertrophic response (i.e. cardiomyocyte area) to Angiotensin (Ang) II (***Figure 8***). Notably, however, the reduced hypertrophic response to NO, but not ANP nor BNP, significantly increased in the presence of BAY 60-7550 (***Figure 8***). This *in vitro* model of cardiomyocyte hypertrophy presents further evidence of the compartmentalization of NO/GC-1/cGMP signaling with PDE2 in murine hearts.

**PDE2 inhibition augments cardiac cGMP, but not cAMP, levels**

To explore the cGMP- and cAMP hydrolyzing activity of PDE2 in healthy and failing hearts, cardiac cyclic nucleotide concentrations were determined in the *in vivo* models. AAC increased cGMP levels modestly (∼50%) in WT animals but these values were markedly enhanced (∼300%) following ACC in the presence of BAY 60-7550 (***Figure 8***), indicating that PDE2 activity is markedly upregulated in HF and plays a central role in curbing cGMP signaling. This pattern of activity was entirely absent in GC-1α$^{-/-}$ hearts but maintained in GC-A$^{-/-}$ hearts (***Figure 8***), paralleling the *in vivo* functional pharmacology. In contrast, BAY 60-7550 was unable to influence global cAMP levels in WT animals with AAC (***Figure 8***), which fits with previous reports of compartmentalized effects of PDE2 inhibition on local pools of cAMP(40) (which would entail a lack of effect on overall cardiac cAMP concentrations); alternatively, PDE2 may not play a key role in cAMP metabolism in this setting.

Finally, the expression of PDE2A was upregulated in hearts from mice undergoing AAC (***Figure 8***), consistent with published data in animal models and patients with HF(39); however, this recognized increase in PDE2A was not as pronounced in the sympathetic hyper-activation model (***SI Appendix, Figure S2***), despite the clear pharmacological benefit of PDE2i; this suggests the trigger(s) for promoting PDE2A expression is specific to pressure overload rather than enhanced sympathetic drive. In addition, PDE2A appeared to exhibit somewhat of a sarcomeric localization (i.e. a striated staining pattern) but this did not change patently in the face of pressure overload, implying that PDE2A localization does not spatially adjust during HF to physically associate with a different cGMP pool (***SI Appendix, Figure S7***).

**PDE2 inhibition reduces the expression and/or activity or a number of pro-hypertrophic and pro-fibrotic signaling pathways**

To glean molecular insight into the downstream pathways coupled to NO/GC-1α signaling in the context of PDE2i in HF we employed quantitative PCR to interrogate a number of established cardiac hypertrophic and fibrotic mediators(59). Here, administration of BAY 60-7550 resulted in reduced expression of a number of pro-fibrotic markers (e.g. Col1α1 and Col1α2) and drivers (e.g. TGFβ, CTGF), albeit without affecting ColIVα1 or fibronectin (***SI Appendix, Figure S8***); this profile matched the strong anti-fibrotic effect observed *in vivo*. The anti-hypertrophic action of PDE2i documented *in vivo* was supported by a significant reduction in ANP and βMHC expression, but this did not appear to occur via significant modulation of established pro-

hypertrophic pathways such as NFAT or GSK3β (***SI Appendix, Figure S8***).

## DISCUSSION

The strategy of promoting cardiac cGMP signaling, by dual neutral endopeptidase/angiotensin receptor blockers or sGC stimulators, is clinically effective in HF(15, 23). Blockade of several PDE isoforms, including PDE1(25), PDE5(27), or PDE9(28), has shown promise in experimental models of HF suggesting targeting these cGMP-hydrolyzing enzymes may also offer therapeutic gain, although a large-scale clinical evaluation of PDE5 inhibition in HFpEF patients did not meet its primary endpoint(37). Previous work has demonstrated positive pharmacology through inhibition of an additional PDE isozyme, PDE2, in restoring pulmonary hemodynamics, vascular remodeling and RVH in preclinical models of PH(46). Increased expression and activity of PDE2 in experimental models(60, 61) and patients with HF(39) provides support for also targeting this isozyme in HF; however, previous studies have largely focused on the cAMP-hydrolyzing capacity of the enzyme, with both positive(44) and negative(39) outcomes. The present study took a functional approach to evaluate the capacity of PDE2 as a cGMP-hydrolyzing enzyme to modulate the pathogenesis of experimental HF. Using both pressure overload and isoproterenol -induced LVH and cardiac dysfunction, we describe that PDE2A expression and activity is up-regulated as a consequence of disease, and that PDE2 inhibition proffers a multi-pronged protective effect. In both models, the decline in contractility, LVH, LV dilatation and fibrotic lesions were halted, and often reversed, by administration of the selective PDE2i BAY 60-7550. This was mirrored, at a more molecular level, by increases in cardiac cGMP (but not cAMP) levels and diminution of a range of hypertrophic and fibrotic markers. These observations suggest PDE2 plays a central role in the reduced cGMP signaling that has been shown to characterize HF, at least in experimental models, leading to misaligned myocardial energetics, compromised cardiac performance, and coronary vascular dysfunction(62). Moreover, that inhibition of this PDE isozyme may represent a novel means with which to promote cardiac cGMP signaling for therapeutic gain.

In order to determine if the beneficial effects of PDE2 inhibition in experimental HF were dependent on cGMP derived from NO- or natriuretic peptide- sensitive GCs, we conducted parallel studies in animals deficient in either cGMP-dependent signaling system. In this setting, the positive effect of BAY 60-7550 on cardiac dysfunction was maintained in GC-A$^{-/-}$ mice (the cognate receptor for ANP and BNP(63)), but absent in animals harboring a genetic deletion of GC-1α. In a logical extension to these findings, studies were repeated in animals treated chronically with the non-selective NOS inhibitor L-NAME; an identical response profile was observed in that the efficacy of BAY 60-7550 apparent in WT mice was completely abrogated. This finding indicates that the up-regulation of PDE2 curtails, specifically, NO/GC-1/cGMP signal transduction to exert a cardioprotective benefit in HF, but does little or nothing to alter natriuretic peptide-triggered pathways which are well-established to be anti-hypertrophic and anti-fibrotic(13, 14, 63). Indeed, this differential mechanism of action is illustrated perfectly in the sympathetic hyperactivation model, where genetic deletion of GC-A$^{-/-}$ resulted in significant mortality (i.e. inherent cardioprotective function of ANP/BNP) but rescued by administration of BAY 60-7550 (i.e. augmenting NO/GC-1 signaling). These data suggest that PDE2 inhibitors may be an effective means by which to promote cardioprotective NO/GC-1/cGMP signaling and offset the deterioration in LV function typical of HF patients.

These data align well with the observation that PDE2 can be allosterically-activated by NO/GC-1-derived cGMP to modulate cAMP bioactivity in cardiac myocytes(40). However, in terms of functional cGMP effects, cell-based studies have intimated that membrane-bound PDE2 is more efficient in hydrolyzing particulate GC-generated cGMP as a result of intracellular compartmentalization(26). Nevertheless, the co-localization of GC-1 with chaperones that promote association with the cytoplasmic membrane (e.g. Hsp70(64) and Hsp90(65)), does provide the rationale for a local pool of GC-1-generated cGMP in close proximity to PDE2. This concept is supported by nNOS translocation to the plasma membrane in failing hearts(66), and that caveolin-binding protects GC-1 from translocation, oxidation and loss of NO-sensitivity in response to volume overload(67, 68). A similar pattern of activity was observed in terms of changes in coronary flow in isolated hearts. Here, BAY 60-7550 caused a modest vasodilatation in hearts from WT and GC-A$^{-/-}$ mice, but this effect was absent in hearts lacking GC-1α; again, this suggests PDE2 blockade enhances signaling by (endothelium-derived) NO/GC-1 in the coronary vasculature. This dilator effect on coronary function, albeit modest, would be a welcome additional action of PDE2 inhibitors in the context of HF since it would promote increased blood flow to the myocardium. Interestingly however, PDE2 inhibition did not augment SNP-induced vasodilatation in the coronary circulation, suggesting it might exert a subtle effect on endothelium-derived NO bioactivity rather than on agonist-stimulated dilatation. Despite the pharmacological benefit of PDE2 inhibition and augmentation of NO/GC-1/cGMP signaling in HF models in WT mice, we noted that genetic deletion of GC-1α did not cause a significant intrinsic deterioration in the cardiac dysfunction associated with AAC. This fits with previous work reporting that eNOS$^{-/-}$, GC-1α$^{-/-}$ and cardiomyocyte-specific G-kinase (cGK)I$^{-/-}$ mice do not exhibit exacerbated LVH in response to pressure overload in the context of hypertension(58, 69) and/or AAC(50, 70) (although more stringent thoracic aortic constriction does tease out an aggravated phenotype(71, 72)). The reason for this apparent disconnect is almost certainly the release of natriuretic peptides upon cardiac stress, which is sufficient to maintain relatively intact cardiac structure and function(69). This observation therefore suggests that NO/GC-1/cGMP signaling does not innately impact the development of LVH and HF, but can be harnessed pharmacologically to provide a therapeutic benefit. This is despite the fact that GC-1α expression tends to be up-regulated in response to pressure overload. The logical explanation for this is that impairment of this signaling cascade, for example through eNOS uncoupling(10), GC-1β heme oxidation(8, 67), and/or up-regulation of PDEs(25, 27, 28), diminishes its influence to such an extent that only under circumstances of pharmacological augmentation can it meaningfully affect disease progression. The downstream signaling pathways driven by PDE2-regulated cGMP pools to bring about this beneficial action on NO/GC-1 signaling warrant further investigation. Involvement of cGKI in the anti-hypertrophic and anti-fibrotic actions of cGMP does not always appear to be a prerequisite (70, 73-75) but the kinase is known to modify well-established targets including $Ca^{2+}$ channels and $Ca^{2+}$ sequestration(17, 18), calcineurin/NFAT signaling(19), RGS (20), transient receptor potential cation channel (TRPC) 6(21) and myosin binding protein (MBP)-C(22). Activation of cGKII might represent an alternate pathway since this cGK isoform in tandem with PDE2 is responsible for governing aldosterone production in the adrenal cortex (76). Reports that PDE2 regulates the cGMP-dependent sympatholytic effects of NO and natriuretic peptides (55, 56) also proffers an intriguing mechanism that might underlie the benefits of PDE2 inhibition in HF.

One explanation for the beneficial effects of PDE2 inhibition on the HF phenotype could be an acute positive inotropic effect via augmentation of cAMP signaling (since this PDE isozyme metabolizes both cGMP and cAMP), which would tend to functionally oppose the failing myocardium. This phenomenon has

been reported previously in mice using BAY 60-7550 or animals over-expressing a cardiac-specific PDE2(43). To test this possibility, isolated hearts from WT, GC-1$\alpha^{-/-}$ and GC-A$^{-/-}$ mice that had undergone pressure-overload or sham surgery were set-up in Langendorff mode. Whilst the lack of sympathetic innervation represents a patent caveat in this setting, it enables examination of more acute responses in the myocardium and coronary vasculature, with sympathetic stimulation being mimicked by administration of isoproterenol. In hearts removed from animals exposed to AAC, myocardial contractility and coronary vascular function were both impaired compared to hearts from sham-operated animals, commensurate with a HF phenotype. Importantly, whilst BAY 60-7550 had a small effect to increase coronary flow in both normal and failing hearts, it was unable to alter any inotropic or chronotropic effects of NO-donors, ANP or isoproterenol. Also, there was no observable change in HR in the presence of BAY 60-7550 (as an index of cardiac cAMP functionality) in either pressure overload or isoproterenol –induced HF. In concert, these observations suggest that PDE2 inhibition has little or no acute effect on cardiac function, even in the face of β-receptor stimulation, and therefore that the beneficial effects we report in experimental HF *in vivo* are longer-term actions on heart structure and the hypertrophic/fibrotic response. Curiously, these observations seem at odds with recent data describing positive chronotropic effects of BAY 60-7550 in mice with acute or chronic β-adrenoceptor activation(43); whether the lower *in vivo* dose in the present study (10 mg/kg/day s.c. v 3 mg/kg bolus i.p.) explains the lack of effect on cAMP signaling remains to be established (even if that is the case it means a tenable window of opportunity exists permitting differentiation between cGMP and cAMP, if required). Conversely, a parallel approach employing transgenic mice over-expressing cardiac-specific PDE2 revealed a significant increase in LV mass at baseline and following MI(43), which would dovetail well with a cardioprotective role for PDE2 underpinned by cGMP.

Recent work has revealed PDE2 to change its substrate profile in the LV based on the dynamic levels of cGMP and cAMP. Thus, under physiological circumstances PDE2 hydrolyses cGMP almost exclusively, whereas in the presence of β-adrenergic activation PDE2 predominantly metabolizes cAMP, thereby restricting adrenergic signaling(26, 40, 61). Our data suggest that PDE2, in experimental models of HF *in vivo* and in isolated hearts *ex vivo*, acts primarily as a 'cGMP-hydrolase'; however, although herein we only provide a cursory inspection, PDE2A localization does not appear to change overtly in response to pressure overload. Yet, this does not rule out an effect of PDE2i on a local pool of cAMP that promotes an anti-hypertrophic activity, as has been recently reported(44); one shortcoming of the present study is that such a confined change would not have been detectable in the whole heart cyclic nucleotide analysis. Indeed, it would be advantageous to target PDE2 in HF if the enzyme regulated generic anti-hypertrophic mechanisms involving both cyclic nucleotides (without an overt effect on contraction/metabolic demand). Interestingly however, these observations are in contrast to contemporary reports describing crosstalk between CNP-triggered cGMP accumulation and cAMP-driven increases in contractility at the level of PDE3 in HF(77). Further investigation is required to determine if the cGMP-elevating activity of PDE2i might indirectly inhibit PDE3 in a similar fashion; again, our assessment of global cyclic nucleotide levels would likely have missed such a compartmentalized effect, but from a functional perspective (i.e. inotropy) this did not seem to be an issue. Additionally, utilization of GC-A$^{-/-}$ mice would not have brought to light an effect on CNP signalling in this context since this natriuretic peptide triggers cGMP generation via GC-B.

The differential activity of NO/GC-1 and natriuretic peptide/GC-A signaling has important implications for the pharmacological manipulation of cGMP for cardioprotective purposes in HF. Ultimately, it seems likely that multiple interventions, targeted at both the NO- and natriuretic peptide–dependent GC/cGMP systems, will provide the optimal pharmacological boost, since whilst there is overlap in the two pathways (e.g. anti-hypertrophic and vasodilator) there are clearly distinct consequences (e.g. anti-platelet versus natriuretic). Thus, it is important that the compartmentalized nature of cGMP (and cAMP) signaling is further delineated with the aim of identifying the most efficient interventions that can achieve this therapeutic goal. Whether this might be inhibiting multiple PDEs, blocking breakdown with concomitant cyclase activation, or most likely parallel activation of NO- and natriuretic peptide– signaling pathways remains to be verified. Herein, the efficacy of PDE2 inhibition is shown to be dependent on intact NO/GC-1/cGMP signaling, as is the case for PDE5 inhibitors(78) (although this esterase may be re-targeted in pathological circumstances(79)). As a consequence, increasing cellular cGMP levels by pharmacological blockade of either PDE2 or PDE5 would be predicted to activate the alternate isozyme as a result of cGMP binding to N-terminal GAF domains possessed by both enzymes(24). This may well explain, at least in part, the lack of efficacy of sildenafil in the RELAX trial, and more positive data in patients would be achieved by dual inhibition of PDE2 and PDE5. However, if both these PDEs are largely constrained to metabolizing NO/GC-1-derived cGMP, then combined blockade of PDE2 and PDE9 (which regulates natriuretic peptide-driven increase in myocardial cGMP(28)) might be superior by promoting NO and natriuretic peptide signaling concomitantly? The caveat here is systemic hypotension as a potentially dose-limiting effect when combining cGMP-elevating agents (and other emerging consequences such as melanoma(80)), so titrating interventions to avoid these concerns would be necessary.

In sum, this study provides convincing evidence *in vitro* and *in vivo* of the therapeutic potential of PDE2 inhibition in LVH and HF in promoting cardioprotective cGMP-signaling. The beneficial effect of PDE2i is dependent on endogenous NO bioactivity and stimulation of GC-1. Thus, PDE2 inhibition, possibly in combination with other cGMP-elevating agents (e.g. PDE5 and/or PDE9 inhibitors) offers a new approach for the treatment of LVH and HF.

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